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Candida boidinii isolates from olive curation water: a promising platform for methanol-based biomanufacturing

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Abstract

Methanol is a promising feedstock for biomanufacturing, but the efficiency of methanol-based bioprocesses is limited by the low rate of methanol utilization pathways and methanol toxicity. Yeast diversity is an attractive biological resource to develop efficient bioprocesses since any effort with strain improvement is more deserving if applied to innate robust strains with relevant catabolic and biosynthetic potential. The present study is in line with such rational and describes the isolation and molecular identification of seven isolates of the methylotrophic species *Candida boidinii* from waters derived from the traditional curation of olives, in different years, and from contaminated superficial soil near fuel stations. The yeast microbiota from those habitats was also characterized. The four *C. boidinii* isolates obtained from the curation of olives' water exhibited significantly higher maximum specific growth rates (range 0.15–0.19 h^{-1}), compared with the three isolates obtained from the fuel contaminated soils (range 0.05–0.06 h^{-1}) when grown on methanol as the sole C-source (1% (v/v), in shake flasks, at 30°C). The isolates exhibit significant robustness towards methanol toxicity that increases as the cultivation temperature decreases from 30°C to 25°C. The better methanol-based growth performance exhibited by *C. boidinii* isolates from olives´ soaking waters could not be essentially attributed to higher methanol tolerance. These methanol-efficient catabolizing isolates are proposed as a promising platform to develop methanol-based bioprocesses.

Keywords Yeast diversity, *Candida boidinii*, Methylotrophic yeasts, Methanol bioconversion, Methanol toxicity, Bio-based economy

Introduction

Among the one-carbon (C1) compounds derived from carbon dioxide (CO_2), methanol is a promising feedstock for biomanufacturing for the transition from a petroleum-based economy to a sustainable bioeconomy (Frazão and Walther 2020; Cotton et al. 2020; Sarp et al. 2021; Zhang et al. 2022a; Mitic et al. 2023). Since it is liquid, methanol is easy to store, and transport compared to gaseous C1 compounds (Cotton et al. 2020). Methanol does not compete with food crops and land being an attractive feedstock in bioprocesses due to its relative low price, renewability, and high availability (Zhang et al. 2018, 2022a; Cotton et al. 2020; Zhan et al. 2021;

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Lee and Sarwar 2022). Methanol can be produced in high quantities from low-quality coal and from CO_2 by photocatalytic or electrical reduction, thus contributing to a nearly zero CO_2 footprint in methanol-based biomanufacturing (Liu et al. 2018a; Rumayor et al. 2019; Fabarius et al. 2021; Sahoo et al. 2021; Kim et al. 2022). Moreover, compared with sugar-based feedstocks, the higher degree of reduction of methanol is beneficial for the production of reduced bioproducts with commercial value (e.g. alcohols, fatty acids, and carboxylic acids) (Sarwar and Lee 2023). Native methylotrophic microorganisms can utilize methanol as the only source of carbon and energy. Although they are ideal hosts for methanol bioconversion and have been examined and engineered to produce value-added bioproducts from methanol, the bioprocess efficiency is restricted by the rate of methanol utilization (Fan et al. 2018; Wang et al. 2023b). This efficiency is limited by the low rate of methanol utilization pathways and by its toxicity. Methanol toxicity (Mota et al. 2021) may limit the productivity of methanol-based bioprocesses (Wang et al. 2020b; Zhan et al. 2021). The toxic effects of this liposoluble molecule are not only due to methanol itself, but also to formaldehyde resulting from methanol bioconversion (Wakayama et al. 2016). Formaldehyde is a DNA-protein crosslinking agent and a branch point in methylotrophic yeasts between the dissimilatory pathway which leads to the generation of CO_2 and the assimilatory pathway, responsible for biomass production (the Xylulose Monophosphate Pathway (XuMP) (Chistoserdova and Kalyuzhnaya 2018; Lee and Sarwar 2022).

Described methylotrophic yeasts belong to a limited number of genera: *Candida*, *Pichia* and some genera that have been more recently separated from *Pichia*, including *Komagataella*, *Kuraishia*, and *Ogataea* (Yurimoto et al. 2011; Yurimoto and Sakai 2019). The most relevant representatives of methylotrophic yeasts are *Candida boidinii*, *Ogataea polymorpha* (syn. *Hansenula polymorpha* or *Pichia angusta*), and *Komagataella phaffii* (syn. *Pichia pastoris*, *Ogataea methanolica* or *Pichia methanolica*) (Hartner and Glieder 2006; Yurimoto and Sakai 2019). Different strategies towards the optimization of methanol catabolism in yeasts have been explored, involving the design of synthetic methylotrophic hosts or the genetic engineering of native methylotrophs (Cai et al. 2022a; Singh et al. 2022; Gan et al. 2023). However, the genetic manipulation of synthetic or native methylotrophic yeasts depends on the availability of efficient genetic engineering tools.

The exploitation of yeast diversity for the identification of natural isolates from different environments is a promising complementary alternative to develop efficient methanol-based bioprocesses. Indeed, any effort with yeast strain improvement is more deserving if applied to innate robust strains with relevant catabolic and biosynthetic potential. The present study is in line with such rational and describes the isolation of efficient methylotrophic yeast strains from two different habitats. They were molecularly identified as belonging to the *Candida boidinii* species and obtained from traditional curation of olives soaking waters or superficial soil contaminated with fuels near two different fuel stations. This isolation process was first designed with the objective of characterizing the yeast microbiota of these samples and to select yeast strains with potential to efficiently utilize glucose and xylose, the two major sugars present in lignocellulosic hydrolysates. The species *C. boidinii* belongs to the Ascomycota phylum, being phylogenetically related to the *Ogataea* clade. *C. boidinii* isolates have been obtained from natural environments (soil, seawater, sap fluxes of many sugar-rich tree species) samples associated with human activities (e.g. wine or olive fermentations) (Leventdurur et al. 2016; Arous et al. 2017; Oliveira et al. 2017). Genome annotated sequences from different *C. boidinii* strains are currently available (Borelli et al. 2016; Camiolo et al. 2017; Krassowski et al. 2018; Shen et al. 2018) being important ant to guide further studies. For all these reasons, the *Candida boidinii* isolates examined in this study are promising hosts for methanol-based bioprocesses.

Materials and methods

Isolation of yeasts

The *Candida boidinii* strains used in this study (indicated in Table 1) were obtained during this work from soaking waters resulting from the traditional curation of olives from Mértola region, Portugal, in different years (IST 350, 473, 509, and 605), or from the superficial layer of soil, collected near two different fuel stations (IST 592, 599, and 600). During the isolation processes, additional yeast isolates from other species were obtained from the samples examined. The isolates *C. boidinii* IST 350 and IST 605 were obtained using ten-fold serial dilutions, in 0.85% (w/v) NaCl, of the original sample of water from the curation of olives. These diluted samples were plated (100 µl) in YPD agar [10 g/L yeast extract (Difco), 20 g/L peptone (Difco), 20 g/L D-glucose (Merck), with 20 g/L agar (NZYTech)]. *C. boidinii* isolates IST 473, 509, 592, 599 and 600, were obtained based on three cycles of culture enrichment (Zaky et al. 2016). Approximately 0.5 mL of the original sample of the soaking waters from the curation of olives (isolates IST 473, and 509) or, in the case of *C. boidinii* isolates IST 592, 599 and 600, one gram of each soil sample, were introduced in 50 mL of growth medium containing 3 g/L malt extract (Sigma-Aldrich), 3 g/L yeast extract (Difco), 5 g/L peptone (Difco), 1 g/L $(NH_4)_2SO_4$ (Panreac), 0.25 g/L KH_2PO (Panreac, pH 5.0) supplemented with 30 g/L of glucose (Scharlau) and 30 g/L of xylose (Sigma-Aldrich), as

Table 1 Yeast isolates obtained from different samples with the correspondent ID, date of sample collection, and isolation methodology

Isolation sample and date	ID	Species	Accession numbers of D1/D2 and ITS consensus sequences	Isolation method
Water from the curation of olives (September 2017)	IST 350 (MUM 24.13)	***Candida boidinii***	OQ064240 [D1/D2] OQ092418 [ITS]	Direct isolation from the original sample on YPD-agar plates.
Water from the curation of olives (September 2019)	IST 472 (MUM 24.15)	*Pichia kluyveri*	PP270051 [D1/D2]	Three enrichment steps. Differential enrichment (3rd) plated onto Zaky's isolation medium supplemented with glucose as the sole C-source.
	IST 473 (MUM 24.16)	***Candida boidinii***	OQ064241 [D1/D2] OQ092419 [ITS]	
Water from the curation of olives (September 2019)	IST 493 (MUM 24.17)	*Pichia membranifaciens*	PP270052[D1/D2]	Three enrichment steps. Differential enrichment (3rd) plated onto Zaky's isolation medium supplemented with xylose as the sole C-source.
	IST 509 (MUM 24.18)	***Candida boidinii***	OQ064242 [D1/D2] OQ092420 [ITS]	
Water from the curation of olives (September 2020)	IST 603	*Candida tropicalis*	PP270053[D1/D2]	Direct isolation from the original sample on YPD-agar plates.
	IST 604	*Wickerhamomyces anomalus*	PP270054[D1/D2]	
	IST 605 (MUM 24.22)	***Candida boidinii***	OQ064246 [D1/D2] OQ092424 [ITS]	
Superficial layer of soil contaminated with fuels (fuel station 1)	IST 592 (MUM 24.19)	***Candida boidinii***	OQ064243 [D1/D2] OQ092421 [ITS]	Three enrichment steps. Differential enrichment (3rd) plated onto Zaky's isolation medium supplemented with glucose as the sole C-source.
	IST 593	*Torulaspora delbrueckii*	PP270055[D1/D2]	
	IST 601	*Meyerozyma caribbica*	PP270056 [D1/D2]	
Superficial layer of soil contaminated with fuels (fuel station 2)	IST 590	*Candida albicans*	PP270057 [D1/D2]	Three enrichment steps. Differential enrichment (3rd) plated onto Zaky's isolation medium supplemented with glucose as the sole C-source.
	IST 591	*Meyerozyma guilliermondii*	PP270058 [D1/D2]	
	IST 597	*Torulaspora quercuum*	PP270059 [D1/D2]	
	IST 598	*Candida albicans*	PP270060 [D1/D2]	
	IST 599 (MUM 24.20)	***Candida boidinii***	OQ064244 [D1/D2] OQ092422 [ITS]	
	IST 600 (MUM 24.21)	***Candida boidinii***	OQ064245 [D1/D2] OQ092423 [ITS]	

D1/D2 and ITS refers to D1/D2 and ITS consensus sequences of the isolated yeasts (D1/D2 for all and D1/D2 and ITS for *C. boidinii*) deposited at the GenBank NCBI database under the listed accession numbers. The deposit numbers in the culture collection MUM (Micoteca of Universidade do Minho, WDCM 816) are also listed for the strains that were relevant to this study

carbon sources. Those growth media were supplemented with chloramphenicol (100 µg/mL) and incubated at 30°C with orbital agitation (150 rpm) for 48 h (first enrichment). Then, 1 mL of the first enrichment cultures was added to 49 mL of the same medium for a second enrichment step. The third enrichment consists of a differential enrichment performed as previously described but using either 60 g/L glucose (IST 592, 599 and 600) or 60 g/L xylose (IST 473 and 509). These media included 3 g/L yeast extract (Difco), 5 g/L peptone (Difco), 1 g/L $(NH_4)_2SO_4$ (Panreac), 0.25 g/L KH_2PO_4 (Panreac), and 20 g/L agar (NZYtech), supplemented with either 60 g/L glucose or 60 g/L xylose (Zaky et al. 2016) as carbon sources, both supplemented with chloramphenicol (100 µg/mL). Plates were incubated at 30°C for 48 h. Isolated colonies were streaked onto fresh YPD agar plates that were incubated under the same conditions to confirm the purity of cultures. Yeast isolates were maintained at 4°C until DNA extraction for molecular identification. For long-term storage, the isolates are preserved at −80°C in YPD medium containing 15% (v/v) glycerol.

Molecular identification of yeast isolates

For the molecular identification of the isolates obtained, genomic DNA was extracted using the phenol: chloroform: isoamyl alcohol method (Hoffman 1997) and used as template for the amplification of the D1/D2 domain sequence of the 26S ribosomal DNA (rDNA) and the internal transcribed spacer (ITS) region of rDNA. Polymerase Chain Reaction (PCR) was performed using the primer pairs NL-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL-4 (5'-GGTCCGTGTTTCAAGACGG-3'), and ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), respectively, considered effective for the taxonomic identification of yeasts (Kurtzman and Robnett 1998). The PCR protocol included a denaturation step at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s,

annealing at 52°C for 20 s, and extension at 72°C for 30 s. A final extension was performed at 72°C for 10 min. To amplify the DNA fragments, the Phusion™ High-Fidelity DNA Polymerase (Thermofisher Scientific) was used. The amplicons from D1/D2 and ITS regions were purified using NZYGel pure (NZYTech) and Sanger-sequencing (Stabvida) using the corresponding primer. The identification was performed by comparing their D1/D2 and ITS sequences with those deposited in GenBank using the BLAST algorithm from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). The consensus sequences from D1/D2 and ITS rDNA regions were deposited in the GenBank under the accession numbers listed in Table 1. All the relevant strains proposed in this study were deposited into the culture collection Micoteca da Universidade do Minho (MUM, WDCM 816) and the deposit numbers are available in Table 1.

Characterization of C. boidinii isolates growth using methanol as the sole carbon source

The preliminary characterization of the ability of the seven *C. boidinii* isolates obtained to catabolize methanol as the sole carbon source, mid-exponential cells pregrown in YPD medium were used to inoculate a medium with methanol as the sole carbon source. This medium was prepared with 6.4 g/L Yeast Nitrogen Base (YNB, from Difco) at pH 6.0, supplemented with 1% (v/v) of methanol. A standardized initial OD_{600nm} of 0.20±0.05 was used. Growth was performed at 30°C with orbital agitation at 250 rpm. Growth curves were followed based on the increase of culture OD_{600nm} for 90 h.

The effect in the growth curve and methanol utilization of increasing the initial methanol concentrations was examined under identical conditions for isolate IST 350 using 1% (v/v), 2% (v/v), 3% (v/v) or 4% (v/v) methanol (VWR), at two incubation temperatures: 25°C and 30°C.

The concentration of methanol present during *C. boidinii* IST 350 cultivation was determined by HPLC (Hitachi LaChrom Elite, Tokyo, Japan), using a column Aminex HPX- 87 H (Bio-Rad, Hercules, CA, USA) coupled with a refractive index detector. Culture samples were centrifuged (9700× g, 3 min) and 100 µl of the supernatant was pipetted into high-performance liquid chromatography (HPLC) vials and diluted with 900 µl of 50 mM H_2SO_4. Ten microliters of each sample were robotically loaded on the column and eluted with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min for 30 min. The column and refractive index detector temperature was set at 50°C, respectively. The concentration of methanol was calculated using a calibration curve.

Maximum specific growth and methanol consumption rates were calculated by least-square fitting to the linear parts of semi-logarithmic (optical density at 600 nm and methanol concentration, respectively) plots versus time.

Methanol- and salt-induced stress susceptibility assays

The tolerance to methanol or to salt of the seven *Candida boidinii* isolates was tested by spot assays. Other isolates obtained from the curation of olives' water, *Pichia kluyveri* IST 472 and *Pichia membranifaciens* IST 493 (Table 1) were also tested for salt tolerance. The highly salt-tolerant species *Debaryomyces hansenii* IST 375 (MUM 24.14) was also used as a positive control. Yeast suspensions were prepared from mid-exponential cell cultures grown in YPD medium. Cells were harvested, washed, and re-suspended in sterile water to a standardized optical density (OD_{600nm}) value of 1.0. Serial 10-fold dilutions (10^0 to 10^4) of these cell suspensions were spotted (4 µL) onto the corresponding solid medium to evaluate salt or methanol tolerance. To assess methanol tolerance, yeast cell suspensions, prepared as described before, were spotted on YPD-agar medium supplemented with 8% (v/v) or 10% (v/v) methanol and plates were incubated at 25°C or 30°C for 48 h. Regarding methanol assimilation efficiency as the sole carbon source (C-source), at increasing methanol concentrations, yeast cell suspensions were spotted on YNB-agar medium supplemented with 1% (v/v) or 6% (v/v) methanol. This spot growth test allowed the assessment of both methanol assimilation efficacy and methanol tolerance. Growth on YNB-agar plates with methanol as C-source was recorded after 90 h of incubation at 25°C or 30°C. To assess salt susceptibility, YPD-agar medium was supplemented with sodium chloride (1.0 M NaCl, Panreac) and yeast cell suspensions were spotted in this medium and plates incubated at 30°C for 72 h. The results shown are representative of equivalent results obtained from at least two independent experiments, following several tests for the selection of the methanol concentrations to be tested.

Results

Isolation and molecular identification of yeast microbiota

The yeast microbiota from the water resulting from traditional curation of olives, collected in different years, and from the superficial layer of soil contaminated with fuels near two different fuel stations, was characterized using the isolation procedures summarized in Table 1. Only ascomycetous yeasts were isolated, including five different genera: *Candida*, *Pichia*, *Wickerhamomyces*, *Torulaspora*, and *Meyerozyma.* The microbiota of the curation of olives' water included the species *Candida boidinii*, *Pichia kluyveri*, *Pichia membranifaciens*, *Candida tropicalis*, and *Wickerhamomyces anomalus* (Table 1) whose isolation from olives has already been reported (Heperkan 2013). From the fuel station superficial soil samples, isolates of *C. boidinii*, *Candida albicans*, *Meyerozyma*

caribbica, *Meyerozyma guilliermondii*, *Torulaspora delbrueckii* and *Torulaspora quercuum* (Table 1), were obtained. The accession numbers of the D1/D2 and ITS consensus sequences of these isolates, molecularly identified at the species level, were deposited at the GenBank NCBI database (Table 1).

Among the yeast species isolated from the two different habitats, only *C. boidinii* is described as methylotrophic (Lachance et al. 2011). Four isolates, molecularly identified as *C. boidinii*, were obtained from the waters resulting from traditional curation of olives, collected in different years: IST 350 in 2017, IST 473 and 509 in 2019, and IST 605 in 2020 (Table 1). Three other *C. boidinii* isolates were obtained from the superficial layer of soil contaminated with fuels, collected near fuel station 1 (IST 592) and fuel station 2 (IST 599, and IST 600) (Table 1). *C. boidinii* isolates IST 350 and IST 605 were isolated directly from the original sample whereas *C. boidinii* IST 473, 509, 592, 599 and 600 isolates were obtained after three cycles of enrichment. The third enrichment step was performed in glucose- (IST 473, 592, 599, 600) or xylose-supplemented (IST 509) medium.

Growth efficiency of *C. boidinii* isolates on methanol as the sole carbon source

The performance of the *C. boidinii* isolates obtained was compared concerning methanol utilization as the sole C-source. The concentration selected for the first comparative analysis was 1% (v/v) methanol. Although low to be used as the sole C-source, this concentration limits methanol toxicity. The ability to grow on methanol as the sole carbon source was confirmed for the seven *C. boidinii* isolates tested (Fig. 1). Remarkably, all the four isolates obtained from the curation of olives´ water in the period 2017–2020 exhibited significantly higher values (around 3-fold) for the maximum specific growth rates (range 0.15–0.19 h^{-1}), compared with all the three isolates obtained from soil near two different fuel stations (range 0.05–0.06 h^{-1}).

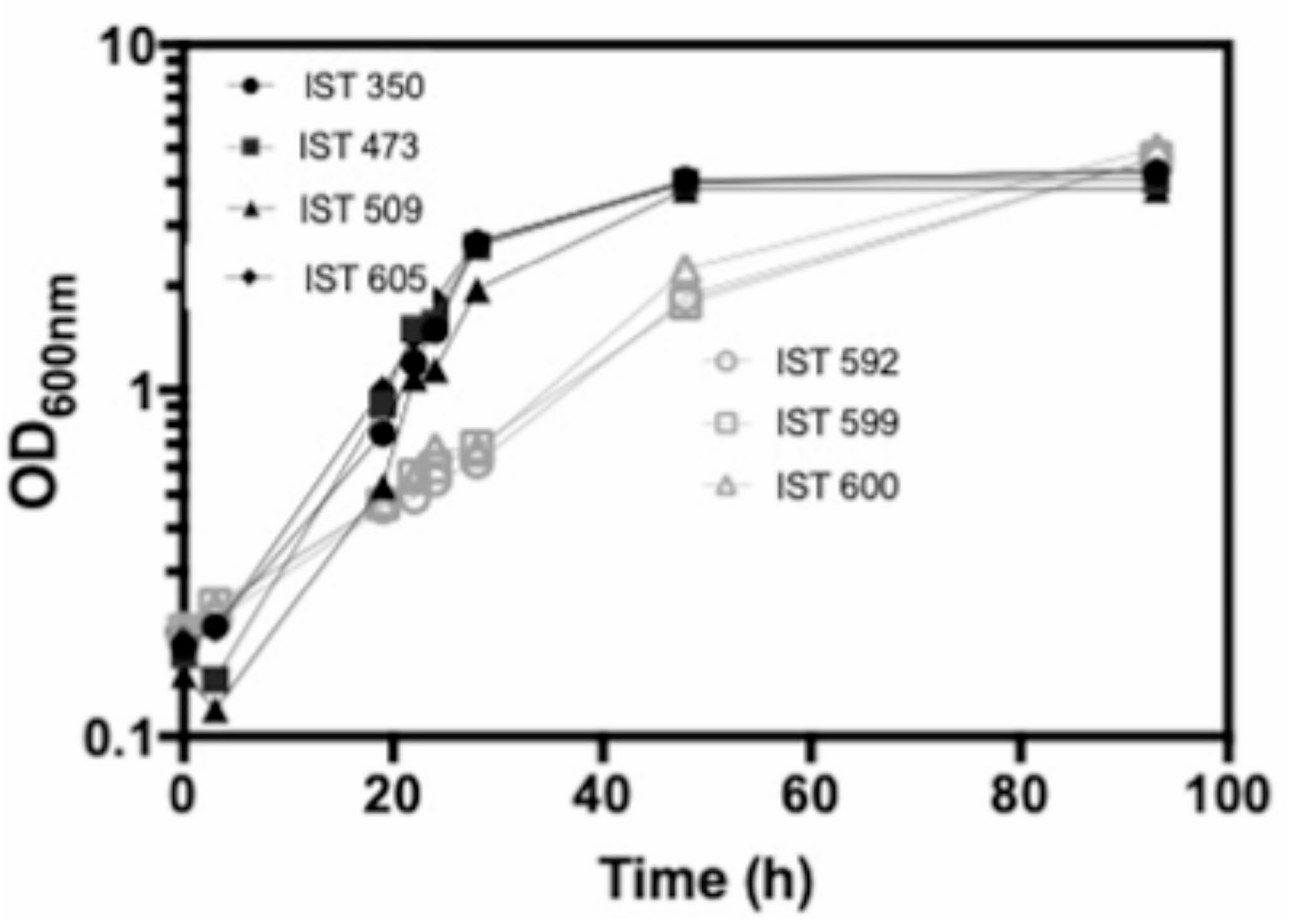

Fig. 1 Growth curves of *Candida boidinii* isolates obtained using 1% (v/v) of methanol as the sole carbon source. The *C. boidinii* isolates obtained in this work from waters from traditional curation of olives in 2017 (IST 350), 2019 (IST 473 and 509) and 2020 (IST 605) or from the superficial layer of soil contaminated near fuel station 1 (IST 592) or station 2 (IST 599 and IST 600) were grown in YNB medium at pH 6.0 supplemented with 1% (v/v) methanol, at 30ºC, in shake flasks with orbital agitation (250 rpm). Growth was followed during 90 h based on culture optical density at 600 nm (OD_{600nm})

For one of the efficient methanol catabolizing isolates obtained from olives curation water, isolate IST 350, the growth curves and methanol utilization profiles were obtained during growth in YNB with increasing initial concentrations of methanol (range 1–4% (v/v)), and at the temperatures 25°C and 30°C. The objective of reducing the cultivation temperature to 25°C was to assess the expected decrease of methanol toxicity given that the optimal and maximum temperatures for growth decrease as the concentration of an inhibitory compound increases (Van Uden 1985; Godinho et al. 2021). For the lower concentration tested (1% (v/v)), methanol was fully catabolized in the first 40 h of cultivation while for higher concentrations of 2% and 3% (v/v) of methanol the cultivation time for complete methanol utilization increased, not surpassing 70 h for 3% (v/v) (Fig. 2). The effect of the temperatures tested on the growth curve was marked for an initial concentration of 4% (v/v). At this high concentration, methanol was not fully consumed at both temperatures, but a clearly higher utilization rate was registered at the lower temperature, 25°C. For lower initial methanol concentrations, a slight improvement of methanol bioconversion at 25ºC was only registered for initial concentrations above 1–2% (v/v), consistent with the balance between the effect of the optimum temperature for growth and methanol tolerance of *C. boidinii* IST 350.

For a closer look on the effect that increasing initial concentrations of methanol had on growth parameters at the incubation temperatures 25°C and 30°C, these values were estimated based on the growth curves shown in Fig. 2(Fig. 3). Incubation at the lower temperature (25°C) apparently led to shorter lag phase duration, this being particularly evident at higher methanol concentrations (Fig. 3, (a)). Regarding the maximum specific growth rate at 25°C for the lower concentration of methanol, the value was below the value calculated at 30ºC and was not affected by the increase of initial methanol concentration up to 2% (v/v) but, for higher concentrations, this value decreased; however, those values were, for all the concentrations tested, above those calculated for growth at 30ºC (Fig. 3, (b)). The maximum specific rate of methanol consumption also suffered a higher methanol inhibition at 30ºC for concentrations above 2% (v/v) (Fig. 3, (c)). The estimated maximum yeast biomass produced, associated to maximum culture OD_{600nm}, increased twice at 25°C as the concentration of methanol increased from 1 to 2% (v/v) methanol (2.1-fold at 25°C, compared with 1.4-fold at 30°C), consistent with the undetectable inhibitory effect of methanol observed at 25°C, for concentrations below 2% (v/v) (Fig. 3, (d)). The differential of the maximum biomass produced at 25°C compared with 30°C increased with the initial methanol concentration, for concentrations above 2% (v/v) with the biomass concentrations attained at 30°C also being below the concentrations attained at 25°C. At the initial concentration of methanol of 4% (v/v), the biomass concentration produced after around 90 h of cultivation, at both temperatures, suffered a marked decrease because this carbon source was not fully catabolized, with a stronger impact at 30ºC (Fig. 3, (d)).

Methanol and salt susceptibility assays of *C. boidinii* isolates

To understand if the higher growth efficiency of the group of *C. boidinii* isolates from the soaking waters from the curation of olives (IST 350, 473, 509, and 605), compared with the group of isolates from superficial layer of soil contaminated with fuels (IST 592, 599, and IST 600), is due to differences in tolerance to methanol or to a more efficient methanol catabolism, methanol susceptibility of the isolates was compared by spot assays in YPD agar plates supplemented with increasing concentrations of methanol (Fig. 4, (a)). The methanol concentrations that had to be used in these spot assays were much higher than those used in liquid medium, possibly because a rich solid medium was used and methanol evaporation during incubation is expected to be higher, even though the growth phenotypes were registered after 48 h of incubation to avoid an exacerbated evaporation. Interestingly, at 1% (v/v) methanol, the less efficient methanol- catabolizing isolates obtained from soil contaminated with biofuels were found to be more tolerant to methanol than some of the isolates obtained from water from olives' curation (Fig. 4, (a)). Remarkably, the isolate IST 350, selected to be examined for growth on increasing concentrations of methanol in shake flasks, exhibits the lower methanol tolerance, indicating that the performance of other isolates from olive curation waters may be better than the registered in Fig. 2 results.

The more efficient growth on methanol of the group of isolates from the water from olives' curation, compared with the other group of isolates, was confirmed by spot assays in YNB medium with methanol as the sole C-source, especially for 1% (v/v) of methanol while such higher efficiency was not so clear for the more toxic methanol concentration of 8% (v/v) (Fig. 4, (b)). Photographs were taken after 90 h of incubation due to slower growth when YNB medium with methanol as the only C-source was used (Fig. 4, (a)).

Regarding salt tolerance, other isolates obtained from the curation of olives' water, *Pichia kluyveri* IST 472 and *Pichia membranifaciens* IST 493 were included in the analysis as well as a strain of the highly salt tolerant species *Debaryomyces hansenii*, as a positive control. As observed for methanol tolerance, the *C. boidinii* isolates obtained from salted water obtained from traditional curation of olives (IST 350, 473, 509, and 605), except for

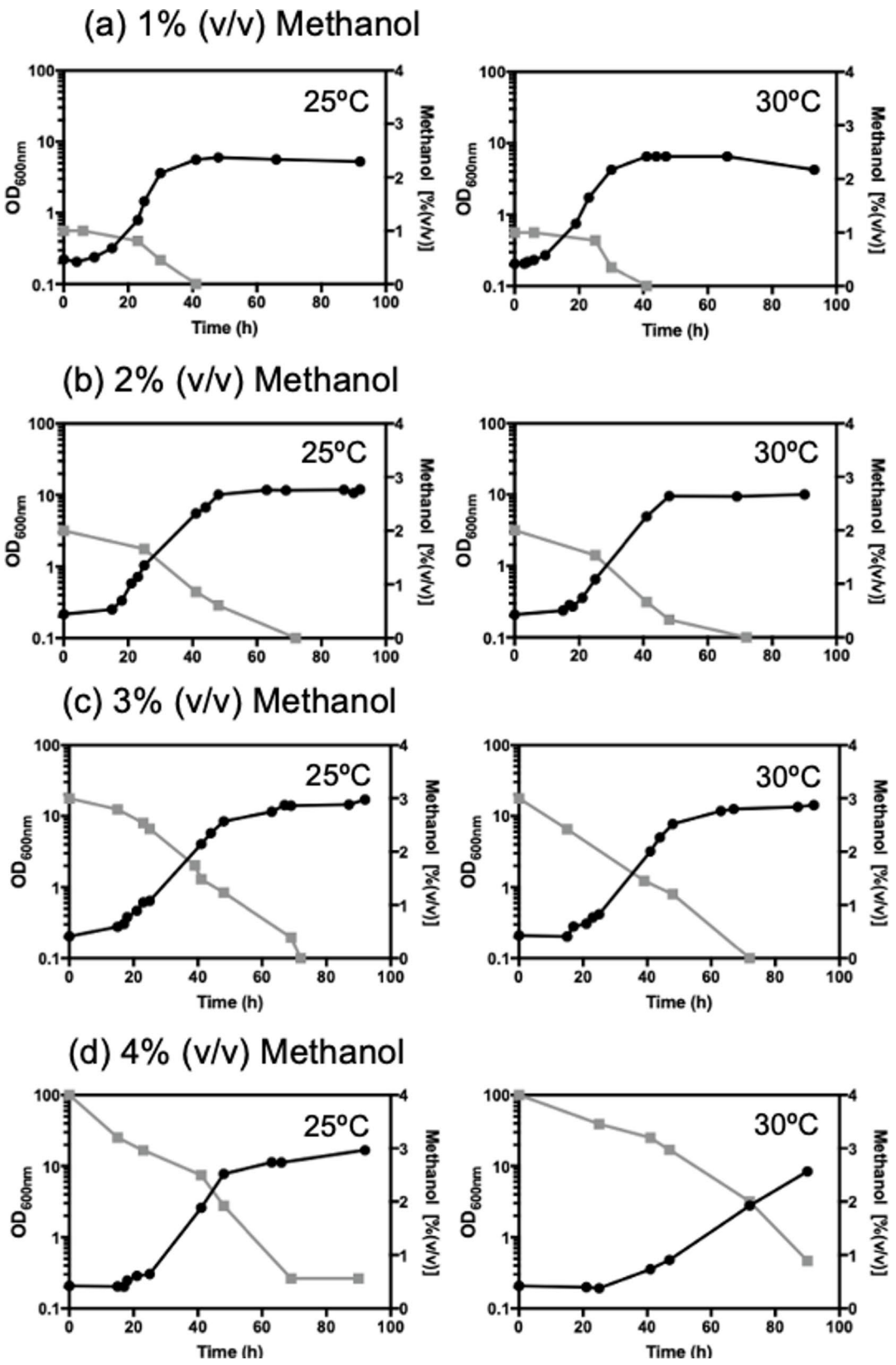

Fig. 2 Growth curves and methanol assimilation by *C. boidinii* IST 350 using increasing initial methanol concentrations**.** Growth curves (black circles) and methanol concentration profiles (grey squares) during cultivation of *C. boidinii* IST 350 in YNB medium supplemented with increasing methanol concentrations of 1% (v/v) **(a)**, 2% (v/v) **(b)**, 3% (v/v) **(c)**, or 4% (v/v) **(d)**, with orbital agitation (250 rpm) at 30 °C. Results are from a representative shake flask experiment of other independent experiments leading to similar results

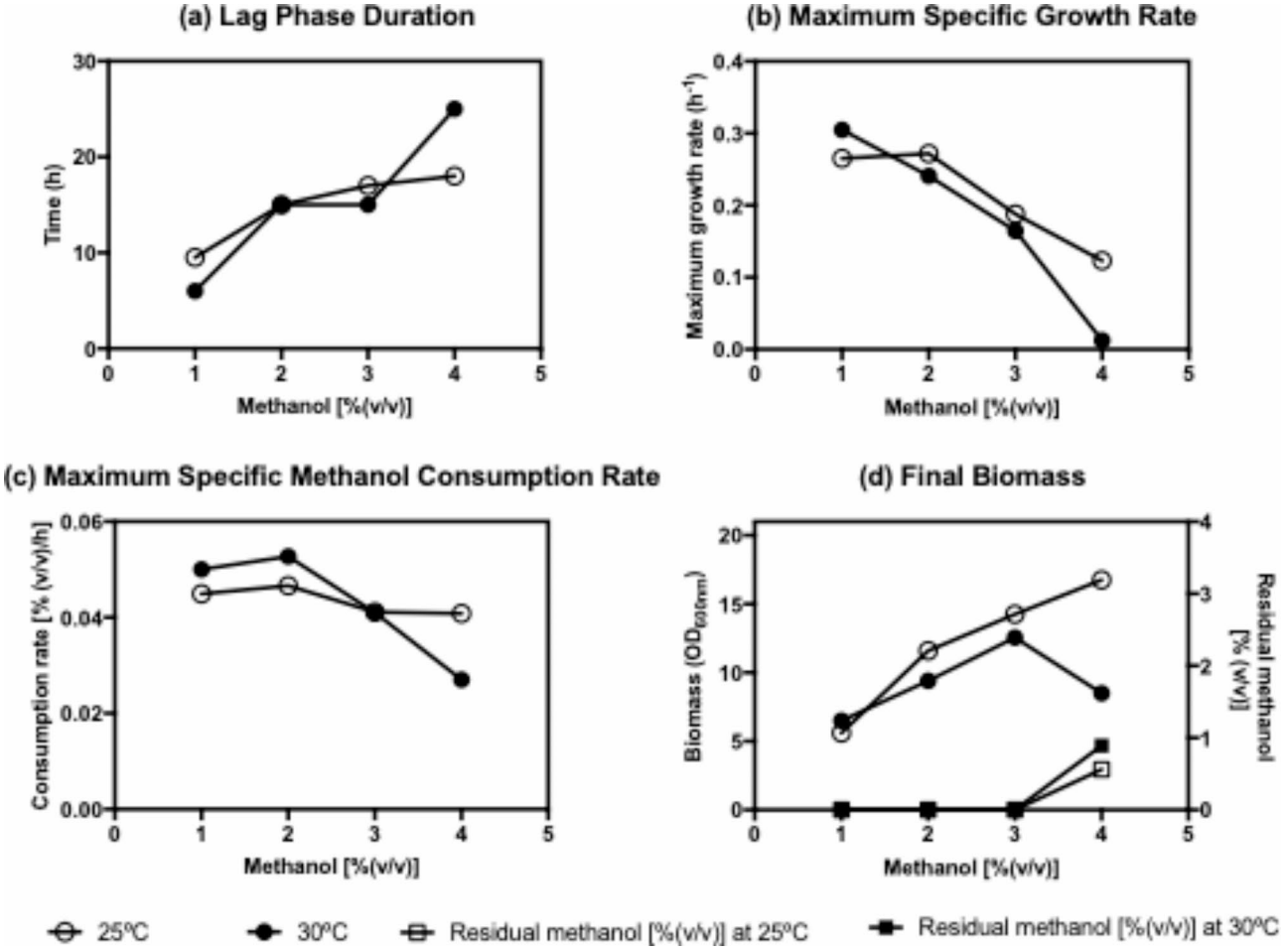

Fig. 3 Effect of increasing initial methanol concentrations on the growth parameters of *C. boidinii* IST 350 incubated in shake flasks at 25°C or 30°C**.** Effect of initial methanol concentration on the duration of the lag phase (**a**), the maximum specific growth rate (**b**) and the maximum specific methanol consumption rate (**c**), and the final biomass produced, associated to culture OD_{600nm} (**d**), of *C. boidinii* IST 350, estimated based on the growth curves shown in Fig. 2. Open symbols are used for cultivations at 25°C and closed symbols at 30 °C. The methanol concentrations present at the end of cultivation are also displayed in the final biomass production panel (squares in (**d**)))

isolate IST 605, were not more tolerant to salt compared with the soil isolates (IST 592, 599, and 600) after 72 h of incubation at 30°C, (Fig. 4, (c)). *P. kluyveri* IST 472 was found to be highly susceptible to saline stress since its growth in YPD-agar medium supplemented with 1.0 M NaCl growth was abrogated whereas *P. membranifaciens* IST 493 isolated from the same salted habitat was highly salt tolerant, similar to *D. hansenii* IST 375. All together, these results appear to suggest that, in general, highly tolerant yeasts are not rigidly selected in the salted habitat of the soaking waters from olives curation.

Discussion

The rich yeast diversity in different ecological environments is a natural resource to be explored for the development of a bio-based economy. Therefore, it is instrumental to obtain strains with diverse useful physiological characteristics such as an efficient catabolism of diverse C- sources, specialized biosynthetic ability to produce value-added bioproducts, and tolerance to a wide range of stresses of industrial relevance. The selection of yeast phenotypes highly suited to specific bioprocesses can be the basis for subsequent effective generation of additional genetic improvement through classic or site-specific mutagenesis, adaptive laboratory evolution, metabolic engineering, and synthetic biology design, depending on the available genetic tools and information for the specific yeast species. Here we propose a group of yeast strains, particularly those isolated from olive curation soaking waters, for methanol-based biomanufacturing. Methanol is a promising feedstock considered as a next-generation feedstock (Zhang et al. 2022a) but using methanol as sole C-source is challenging due to the inefficiency of methylotrophic microorganisms in its assimilation rate into biomass and bioproducts (Zhang et al. 2022a, b). Consistent with the characterization of

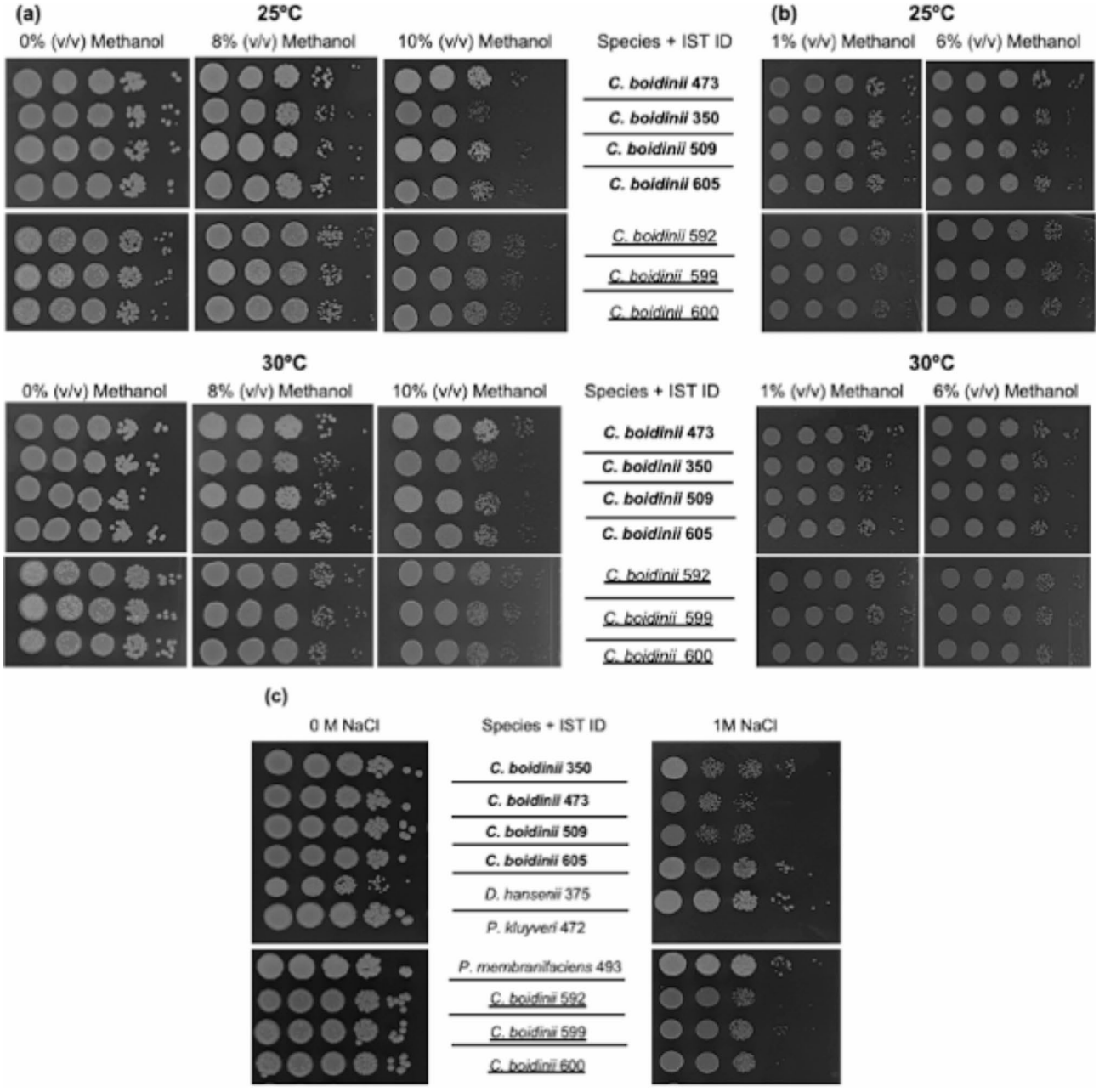

Fig. 4 Susceptibility to methanol- or saline- induced stresses, by spot assays, of the seven *C. boidinii* isolates**.** Spot growth of *C. boidinii* isolates obtained from water from the curation of olives (in bold) or from soil near fuel stations (underlined) in YPD-agar (**a**) or in YNB-agar (**b**) supplemented with increasing concentrations of methanol, used as toxicant (**a**) or as the sole carbon source (**b**), or in YPD-agar supplemented with a high salt (NaCl) concentration (**c**). For comparative analysis, the highly salt-tolerant yeast *Debaryomyces hansenii* IST 375 was used as positive control. Salt susceptibility of *Pichia kluyveri* IST 472 and *Pichia membranifaciens* IST 493, obtained from the same samples of soaking water from olives' curation, were also tested. Registration of phenotypes was performed after incubation for 48 h (**a**) or 90 h (**b**) at 25 °C or 30 °C or for 72 h of growth at 30 °C (**c**)

Candida boidinii as methylotrophic (Hartner and Glieder 2006; Yurimoto et al. 2011), all the seven *C. boidinii* isolates obtained were capable to grow with methanol as the sole carbon and energy source. Remarkably, all the strains isolated from the traditional curation of olives soaking waters, in different years, showed more promising results for highly efficient biomass production from moderate concentration of methanol, compared to those isolated from the superficial layer of soil contaminated with fuels.

The soaking waters resulting from the curation of olives are unique habitats due its low concentration of sugar and relatively high concentrations of oil, phenolic compounds and salt (Arroyo-López et al. 2008; Mujdeci and Ozbas 2021). Together with *P. kluyveri*, *P.*

membranifaciens and *W. anomalus* (Arroyo-López et al. 2008; Perpetuini et al. 2020), *C. boidinii* was reported to be one of the predominant yeasts in olive fermentation, having a strong lipase activity that contributes to the final organoleptic characteristics of this fruit by changing the olives' free fatty acids composition (Bonatsou et al. 2018; Perpetuini et al. 2020). Interestingly, among all the yeast species of the isolates obtained in this study from the curation of olives´ water, *C. boidinii*, *Candida tropicalis*, *Meyerozyma guilliermondii* and *Wickerhamomyces anomalus*, are described in the literature as oleaginous (Ramírez-Castrillón et al. 2017; Fabricio et al. 2019; Abeln and Chuck 2021; Mota et al. 2022).

The isolate *C. boidinii* IST 350 obtained from those soaking waters exhibit maximum specific growth rates when grown in YNB with methanol as the sole carbon source in shake flasks above those reported for methylotrophic yeasts (Brinkmann et al. 1990; Tomàs-Gamisans et al. 2018; Wefelmeier et al. 2023) and comparable with some methylotrophic bacteria (Šmejkalová et al. 2010; Simões et al. 2022). The apparent lower methanol tolerance exhibited by this isolate, compared with others isolated from the same habitat, in different years, suggests that there is still space to improve methanol-based growth by exploring other bioresources gathered in this work, especially under optimized growth conditions in bioreactors. Initial methanol concentrations in the range 2–3% (v/v) led to the best results for this strain in the range of cultivation temperatures 25–30°C being more favorable at 25°C likely due to reduced methanol toxicity (Sá-Correia and Van Uden 1983; Van Uden 1985). The use of the lower temperature tested (25°C) favors growth and methanol bioconversion compared with 30ºC, and the effect is more evident with the increase of the initial methanol concentration. This effect includes the reduction of the duration of methanol-induced lag phase and the increase of biomass yield and maximum specific growth and methanol consumption rates, emphasizing the described effect of lipophilic compounds in decreasing the optimal and maximum temperature of growth in yeasts (Sá-Correia and Van Uden 1983; Van Uden 1985). In fact, the major bottleneck in methanol-based biomanufacturing is the limited methanol assimilation also related with methanol susceptibility of the majority of the methylotrophic microorganisms (Wang et al. 2023b).

The robustness towards methanol of the *C. boidinii* isolates obtained from the different habitats examined was found to be significant and the much better performance of those isolated from olives curation waters, compared with those isolated from soil contaminated with fuels, could not be related with a higher methanol tolerance but, apparently, with a higher assimilation capacity, at least for moderate methanol concentrations. The methylotrophic yeast *C. boidinii* has been found associated with plant leaves (Kawaguchi et al. 2011) and in other pectin-rich sources, including fruits and wines (Nakagawa et al. 2000). *C. boidinii* exhibits a pectinolytic activity, being able to degrade pectin at the methyl ester moiety using extracellular pectin methylesterases and to utilize this methanol and D-galacturonic acid, the main component of pectin, as C-sources (Nakagawa et al. 2000). Among the yeast species isolated from the curation of olives´ water, C. *boidinii* is the only methylotrophic species (Kurtzman 2011a, b; Lachance et al. 2011). Since the methanol concentrations found in these environments are relatively low (Sánchez et al. 2000; Montaño et al. 2003), it is likely that a high methanol tolerance is not a dominant trait among the methylotrophic yeast isolates present while methanol-based growth efficiency should be to enhance the assimilation of pectic substances as well as competitiveness and adaptation to this specific habitat (Stratilová et al. 1998). However, methanol assimilation demands higher maintenance energy compared to growth on glucose (Tomàs-Gamisans et al. 2018; Guo et al. 2023), partly due to the low energy efficiency of the Xylulose monophosphate (XuMP) pathway. In this pathway, one molecule of ATP is consumed for each pyruvate molecule produced while in glycolysis one molecule of ATP is generated per pyruvate molecule (Guo et al. 2023). Furthermore, a significant portion of the assimilated carbon is oxidized to carbon dioxide in the dissimilatory branch of methanol metabolism (Jordà et al. 2014). Although methylotrophs are ideal hosts for methanol bioconversion and have been examined and engineered to produce value-added bioproducts from methanol, the bioprocess efficiency is restricted by the rate of methanol utilization (Fan et al. 2018; Wang et al. 2023b). This efficiency is limited by the low rate of methanol utilization pathways and by methanol toxicity. Attempts to improve methanol catabolism in the methylotrophic yeast *Pichia pastoris (*now *Komagataella phaffii*) included the rewiring of central metabolism for efficient production of free fatty acids from methanol (Cai et al. 2022c) or the reduction of alcohol oxidase activity and addition of sodium citrate (Liu et al. 2023), previously described to improve glutathione synthesis (Gao et al. 2022), and the stimulation of the activity of isocitrate dehydrogenase and strengthening of the tricarboxylic acid cycle (Chen et al. 2016). The supplementation of growth media with yeast extract, amino acids or vitamins was also found to be beneficial to cell growth and metabolism, compensating the cytotoxicity and low energy content of methanol(Zhang et al. 2022a) and the limited supply of amino acids, nucleotide sugars and acetyl-CoA that are the precursors for the biosynthetic machinery (Cai et al. 2021; Zhang et al. 2022a; Wegat et al. 2022; Wang et al. 2023a).

C. boidinii is considered an oleaginous yeast suitable for lipid production for biodiesel either derived from glycerol (Papanikolaou et al. 2017) or pumpkin peel hydrolysate (Demiray et al. 2022) substrate. However, although under non-optimized conditions, preliminary results suggest that methanol-based lipid production by the isolates obtained in this work does not allow lipid contents above 10% (v/v). Nevertheless, the engineering of nativ methylotrophs to produce increased lipid concentration has potential, as is the case of other bioproducts. Interesting examples include the production of malic acid (Guo et al. 2021; Wefelmeier et al. 2024), lactic acid (Wefelmeier et al. 2023), isoprene and acetone (Wefelmeier et al. 2024), β-alanine (Miao et al. 2021), lovastatin (Liu et al. 2018b), long-chain α-alkenes (Cai et al. 2022b). The availability of genome annotated sequences from different *C. boidinii* strains(Borelli et al. 2016; Camiolo et al. 2017; Krassowski et al. 2018; Shen et al. 2018) pave the way for the rational genetic manipulation of the *C. boidinii* isolates obtained in this work as robust and efficient hosts for methanol biomanufacturing processes. If the necessary efficient genome engineering tools are not available or can be easily developed, the adaptive laboratory evolution (ALE) strategy is a promising alternative (Sandberg et al. 2019; Arora et al. 2020; Wang et al. 2023b). There are examples of the successful use of ALE to obtain evolved methanol tolerant strains, as in the case of the non-methylotrophic oleaginous yeast *Rhodotorula toruloides* (Fernandes et al. 2023), or to increase methanol assimilation in non-native methylotroph bacteria, such as *Corynebacterium glutamicum* (Wang et al. 2020a) or *Escherichia coli* (Bennett et al. 2021), or even to stimulate methanol catabolism in *S. cerevisiae* (Espinosa et al. 2020). Concerning methylotrophs, the genetic engineering of the native methylotrophic yeast *Ogataea polymorpha*, combining adaptive laboratory evolution, rational metabolic engineering and multi-omics analyses was a successful example of an efficient production of free fatty acids using methanol as the sole carbon source (Zhang et al. 2022b). These examples emphasize the relatively unexplored potential of optimization of native methylotrophs as robust cell factories and the results obtained in this study highlight the potential of the *C. boidini* isolates obtained towards the development of efficient methanol conversion bioprocesses.

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Author contributions
MNM, MP, and ISC conceived and designed the experiments. MNM, and MP carried out the experimental work. MNM prepared the figures and contributed to the writing of the manuscript under the scientific supervision of ISC, who coordinated the study. All authors read and approved the final manuscript.

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Data availability
The authors confirm that all this study data is available within the article. The relevant isolates were deposited into the Micoteca da Universidade do Minho (MUM) Culture Collection (WDCM 816).

Declarations

Conflict of interest
The authors declare no competing interests.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

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References

Abeln F, Chuck CJ (2021) The history, state of the art and future prospects for oleaginous yeast research. Microb Cell Fact 20:1–31. https://doi.org/10.1186/s12934-021-01712-1

Arora N, Yen HW, Philippidis GP (2020) Harnessing the power of mutagenesis and adaptive laboratory evolution for high lipid production by oleaginous microalgae and yeasts. Sustain (Switzerland). https://doi.org/10.3390/su12125125

Arous F, Azabou S, Triantaphyllidou IE, Aggelis G, Jaouani A, Nasri M, Mechichi T (2017) Newly isolated yeasts from Tunisian microhabitats: lipid accumulation and fatty acid composition. Eng Life Sci 17:226–236. https://doi.org/10.1002/elsc.201500156

Arroyo-López FN, Querol A, Bautista-Gallego J, Garrido-Fernández A (2008) Role of yeasts in table olive production. Int J Food Microbiol 128:189–196. https://doi.org/10.1016/j.ijfoodmicro.2008.08.018

Bennett RK, Gregory GJ, Gonzalez JE, Har JRG, Antoniewicz MR, Papoutsakis ET (2021) Improving the methanol tolerance of an *Escherichia coli* methylotroph via adaptive laboratory evolution enhances synthetic methanol utilization. Front Microbiol 12:1–11. https://doi.org/10.3389/fmicb.2021.638426

Bonatsou S, Paramithiotis S, Panagou EZ (2018) Evolution of yeast consortia during the fermentation of Kalamata natural black olives upon two initial acidification treatments. Front Microbiol 8:1–13. https://doi.org/10.3389/fmicb.2017.02673

Borelli G, José J, Teixeira PJPL, dos Santos LV, Pereira GAG (2016) De novo assembly of *Candida sojae* and *Candida boidinii* genomes, unexplored xylose-consuming yeasts with potential for renewable biochemical production. Genome Announc 4:1–2. https://doi.org/10.1128/genomeA.01551-15

Brinkmann U, Mueller RH, Babel W (1990) The growth rate-limiting reaction in methanol-assimilating yeasts. FEMS Microbiol Lett 87:261–265. https://doi.org/10.1016/0378-1097(90)90464-2

Cai HL, Doi R, Shimada M, Hayakawa T, Nakagawa T (2021) Metabolic regulation adapting to high methanol environment in the methylotrophic yeast *Ogataea methanolica*. Microb Biotechnol 14:1512–1524. https://doi.org/10.1111/1751-7915.13811

Cai HL, Shimada M, Nakagawa T (2022a) The potential and capability of the methylotrophic yeast *Ogataea methanolica* in a methanol bioeconomy. Yeast 39:440–448. https://doi.org/10.1002/yea.3807

Cai P, Li Y, Zhai X, Yao L, Ma X, Jia L, Zhou YJ (2022b) Microbial synthesis of long-chain α-alkenes from methanol by engineering *Pichia pastoris*. Bioresour Bioprocess 9:1–8. https://doi.org/10.1186/s40643-022-00551-1

Cai P, Wu X, Deng J, Gao L, Shen Y, Yao L, Zhou YJ (2022c) Methanol biotransformation toward high-level production of fatty acid derivatives by engineering

the industrial yeast *Pichia pastoris*. Proc Natl Acad Sci U S A 119:1–9. https://doi.org/10.1073/pnas.2201711119
Camiolo S, Porru C, Benítez-Cabello A, Rodríguez-Gómez F, Calero-Delgado B, Porceddu A, Budroni M, Mannazzu I, Jiménez-Díaz R, Arroyo-López FN (2017) Genome overview of eight *Candida boidinii* strains isolated from human activities and wild environments. Stand Genomic Sci 12:1–14. https://doi.org/10.1186/s40793-017-0281-z
Chen H, Wang Z, Wang Z, Dou J, Zhou C (2016) Improving methionine and ATP availability by *MET6* and *SAM2* co-expression combined with sodium citrate feeding enhanced SAM accumulation in *Saccharomyces cerevisiae*. World J Microbiol Biotechnol 32:1–10. https://doi.org/10.1007/s11274-016-2010-y
Chistoserdova L, Kalyuzhnaya MG (2018) Current trends in methylotrophy. Trends Microbiol 26:703–714. https://doi.org/10.1016/j.tim.2018.01.011
Cotton CA, Claassens NJ, Benito-Vaquerizo S, Bar-Even A (2020) Renewable methanol and formate as microbial feedstocks. Curr Opin Biotechnol 62:168–180. https://doi.org/10.1016/j.copbio.2019.10.002
Demiray E, Karatay SE, Dönmez G (2022) Optimization study for enhanced biodiesel production by novel yeast isolates cultivated in dilute acid pretreated Pumpkin Peel. Bioenergy Res 15:1472–1481. https://doi.org/10.1007/s12155-022-10483-5
Espinosa MI, Gonzalez-Garcia RA, Valgepea K, Plan MR, Scott C, Pretorius IS, Marcellin E, Paulsen IT, Williams TC (2020) Adaptive laboratory evolution of native methanol assimilation in *Saccharomyces cerevisiae*. Nat Commun 11:5564. https://doi.org/10.1038/s41467-020-19390-9
Fabarius JT, Wegat V, Roth A, Sieber V (2021) Synthetic methylotrophy in yeasts: towards a circular bioeconomy. Trends Biotechnol 39:348–358. https://doi.org/10.1016/j.tibtech.2020.08.008
Fabricio MF, Valente P, Záchia Ayub MA (2019) Oleaginous yeast *Meyerozyma guilliermondii* shows fermentative metabolism of sugars in the biosynthesis of ethanol and converts raw glycerol and cheese whey permeate into polyunsaturated fatty acids. Biotechnol Prog 35:1–8. https://doi.org/10.1002/btpr.2895
Fan L, Wang Y, Tuyishime P, Gao N, Li Q, Zheng P, Sun J, Ma Y (2018) Engineering artificial fusion proteins for enhanced methanol Bioconversion. ChemBioChem 19:2465–2471. https://doi.org/10.1002/cbic.201800424
Fernandes MA, Mota MN, Faria NT, Sá-Correia I (2023) An evolved strain of the oleaginous yeast *Rhodotorula toruloides*, multi-tolerant to the major inhibitors present in lignocellulosic hydrolysates, exhibits an altered cell envelope. J Fungi. https://doi.org/10.3390/jof9111073
Frazão CJR, Walther T (2020) Syngas and methanol-based biorefinery concepts. Chem Ing Tech 92:1680–1699. https://doi.org/10.1002/cite.202000108
Gan Y, Meng X, Gao C, Song W, Liu L, Chen X (2023) Metabolic engineering strategies for microbial utilization of methanol. Eng Microbiol 3:100081. https://doi.org/10.1016/j.engmic.2023.100081
Gao Y, Liu N, Zhu Y, Yu S, Liu Q, Shi X, Xu J, Xu G, Zhang X, Shi J, Xu Z (2022) Improving glutathione production by engineered *Pichia pastoris*: strain construction and optimal precursor feeding. Appl Microbiol Biotechnol 106:1905–1917. https://doi.org/10.1007/s00253-022-11827-z
Godinho CP, Costa R, Sá-Correia I (2021) The ABC transporter Pdr18 is required for yeast thermotolerance due to its role in ergosterol transport and plasma membrane properties. Environ Microbiol 23:69–80. https://doi.org/10.1111/1462-2920.15253
Guo F, Dai Z, Peng W, Zhang S, Zhou J, Ma J, Dong W, Xin F, Zhang W, Jiang M (2021) Metabolic engineering of *Pichia pastoris* for malic acid production from methanol. Biotechnol Bioeng 118:357–371. https://doi.org/10.1002/bit.27575
Guo F, Qiao Y, Xin F, Zhang W, Jiang M (2023) Bioconversion of C1 feedstocks for chemical production using *Pichia pastoris*. Trends Biotechnol 41:1066–1079. https://doi.org/10.1016/j.tibtech.2023.03.006
Hartner FS, Glieder A (2006) Regulation of methanol utilisation pathway genes in yeasts. Microb Cell Fact 5:1–21. https://doi.org/10.1186/1475-2859-5-39
Heperkan D (2013) Microbiota of table olive fermentations and criteria of selection for their use as starters. Front Microbiol 4:1–11. https://doi.org/10.3389/fmicb.2013.00143
Hoffman CS (1997) Preparation of yeast DNA. Curr Protoc Mol Biol 39:11–14. https://doi.org/10.1002/0471142727.mb1311s39
Jordà J, De Jesus SS, Peltier S, Ferrer P, Albiol J (2014) Metabolic flux analysis of recombinant *Pichia pastoris* growing on different glycerol/methanol mixtures by iterative fitting of NMR-derived 13 C-labelling data from proteinogenic amino acids. N Biotechnol 31:120–132. https://doi.org/10.1016/j.nbt.2013.06.007
Kawaguchi K, Yurimoto H, Oku M, Sakai Y (2011) Yeast methylotrophy and autophagy in a methanol-oscillating environment on growing *Arabidopsis thaliana* leaves. PLoS ONE 6:1–9. https://doi.org/10.1371/journal.pone.0025257
Kim H, Byun M, Lee B, Lim H (2022) Carbon-neutral methanol synthesis as carbon dioxide utilization at different scales: economic and environmental perspectives. Energy Convers Manag 252:115119. https://doi.org/10.1016/j.enconman.2021.115119
Krassowski T, Coughlan AY, Shen XX, Zhou X, Kominek J, Opulente DA, Riley R, Grigoriev IV, Maheshwari N, Shields DC, Kurtzman CP, Hittinger CT, Rokas A, Wolfe KH (2018) Evolutionary instability of CUG-Leu in the genetic code of budding yeasts. Nat Commun 9:1–8. https://doi.org/10.1038/s41467-018-04374-7
Kurtzman CP (2011a) *Pichia* E.C: Hansen (1904). Elsevier B.V.
Kurtzman CP (2011b) *Wickerhamomyces* Kurtzman, Robnett & Basehoar-Powers (2008). Elsevier B.V
Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73:331–371. https://doi.org/10.1023/A:1001761008817
Lachance MA, Boekhout T, Scorzetti G, Fell JW, Kurtzman CP (2011) *Candida* Berkhout (1923). The Yeasts 2:987–1278. https://doi.org/10.1016/B978-0-444-52149-1.00090-2
Lee EY, Sarwar A (2022) Methanol-tolerant yeast for biofuel production. Nat Metab 4:800–801. https://doi.org/10.1038/s42255-022-00603-y
Leventdurur S, Sert-Aydın S, Boyaci-Gunduz CP, Agirman B, Ben Ghorbal A, Francesca N, Martorana A, Erten H (2016) Yeast biota of naturally fermented black olives in different brines made from cv. Gemlik grown in various districts of the Cukurova region of Turkey. Yeast 33:289–301. https://doi.org/10.1002/yea.3170
Liu WC, Baek J, Somorjai GA (2018a) The methanol economy: methane and carbon dioxide conversion. Top Catal 61:530–541. https://doi.org/10.1007/s11244-018-0907-4
Liu Y, Bai C, Xu Q, Yu J, Zhou X, Zhang Y, Cai M (2018b) Improved methanol-derived lovastatin production through enhancement of the biosynthetic pathway and intracellular lovastatin efflux in methylotrophic yeast. Bioresour Bioprocess. https://doi.org/10.1186/s40643-018-0202-z
Liu S, Dong H, Hong K, Meng J, Lin L, Wu X (2023) Improving methanol utilization by reducing alcohol oxidase activity and adding co-substrate of Sodium citrate in *Pichia pastoris*. J Fungi 9:1–16. https://doi.org/10.3390/jof9040422
Miao L, Li Y, Zhu T (2021) Metabolic engineering of methylotrophic *Pichia pastoris* for the production of β-alanine. Bioresour Bioprocess. https://doi.org/10.1186/s40643-021-00444-9
Mitic BM, Troyer C, Lutz L, Baumschabl M, Hann S, Mattanovich D (2023) The oxygen-tolerant reductive glycine pathway assimilates methanol, formate and CO2 in the yeast *Komagataella phaffii*. Nat Commun 14:7754. https://doi.org/10.1038/s41467-023-43610-7
Montaño A, Sánchez AH, Casado FJ, De Castro A, Rejano L (2003) Chemical profile of industrially fermented green olives of different varieties. Food Chem 82:297–302. https://doi.org/10.1016/S0308-8146(02)00593-9
Mota MN, Martins LC, Sá-Correia I (2021) The identification of genetic determinants of methanol tolerance in yeast suggests differences in methanol and ethanol toxicity mechanisms and candidates for improved methanol tolerance engineering. J Fungi 7:1–25. https://doi.org/10.3390/jof7020090
Mota MN, Múgica P, Sá-Correia I (2022) Exploring yeast diversity to produce lipid-based biofuels from agro-forestry and industrial organic residues. J Fungi 8:1–46. https://doi.org/10.3390/jof8070687
Mujdeci GN, Ozbas ZY (2021) Technological and enzymatic characterization of the yeasts isolated from natural fermentation media of Gemlik olives. J Appl Microbiol 131:801–818. https://doi.org/10.1111/jam.14979
Nakagawa T, Miyaji T, Yurimoto H, Sakai Y, Kato N, Tomizuka N (2000) A methylotrophic pathway participates in pectin utilization by *Candida boidinii*. Appl Environ Microbiol 66:4253–4257. https://doi.org/10.1128/AEM.66.10.4253-4257.2000
Oliveira T, Ramalhosa E, Nunes L, Pereira JA, Colla E, Pereira EL (2017) Probiotic potential of indigenous yeasts isolated during the fermentation of table olives from Northeast of Portugal. Innovative Food Sci Emerg Technol 44:167–172. https://doi.org/10.1016/j.ifset.2017.06.003
Papanikolaou S, Rontou M, Belka A, Athenaki M, Gardeli C, Mallouchos A, Kalantzi O, Koutinas AA, Kookos IK, Zeng AP, Aggelis G (2017) Conversion of biodiesel-derived glycerol into biotechnological products of industrial significance by yeast and fungal strains. Eng Life Sci 17:262–281. https://doi.org/10.1002/elsc.201500191

Perpetuini G, Prete R, Garcia-Gonzalez N, Alam MK, Corsetti A (2020) Table olives more than a fermented food. Foods 9:1–16. https://doi.org/10.3390/foods9020178

Ramírez-Castrillón M, Jaramillo-Garcia VP, Rosa PD, Landell MF, Vu D, Fabricio MF, Ayub MAZ, Robert V, Henriques JAP, Valente P (2017) The oleaginous yeast *Meyerozyma guilliermondii* BI281A as a new potential biodiesel feedstock: selection and lipid production optimization. Front Microbiol. https://doi.org/10.3389/fmicb.2017.01776

Rumayor M, Dominguez-Ramos A, Irabien A (2019) Innovative alternatives to methanol manufacture: carbon footprint assessment. J Clean Prod 225:426–434. https://doi.org/10.1016/j.jclepro.2019.03.015

Sá-Correia I, Van Uden N (1983) Temperature profiles of ethanol tolerance: effects of ethanol on the minimum and the maximum temperatures for growth of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. Biotechnol Bioeng 25:1665–1667. https://doi.org/10.1002/bit.260250620

Sahoo KK, Goswami G, Das D (2021) Biotransformation of methane and carbon dioxide into high- value products by methanotrophs: current state of art and future prospects. Front Microbiol 12:1–9. https://doi.org/10.3389/fmicb.2021.636486

Sánchez AH, De Castro A, Rejano L, Montaño A (2000) Comparative study on chemical changes in olive juice and brine during green olive fermentation. J Agric Food Chem 48:5975–5980. https://doi.org/10.1021/jf000563u

Sandberg TE, Salazar MJ, Weng LL, Palsson BO, Feist AM (2019) The emergence of adaptive laboratory evolution as an efficient tool for biological discovery and industrial biotechnology. Metab Eng 56:1–16. https://doi.org/10.1016/j.ymben.2019.08.004

Sarp S, Gonzalez Hernandez S, Chen C, Sheehan SW (2021) Alcohol production from carbon dioxide: methanol as a fuel and chemical feedstock. Joule 5:59–76. https://doi.org/10.1016/j.joule.2020.11.005

Sarwar A, Lee EY (2023) Methanol-based biomanufacturing of fuels and chemicals using native and synthetic methylotrophs. Synth Syst Biotechnol 8:396–415. https://doi.org/10.1016/j.synbio.2023.06.001

Shen X, Opulente DA, Kominek J, Zhou X, Steenwyk JL, Buh KV, Haase MAB, Wisecaver JH, Wang M, Doering DT, Boudouris JT, Schneider RM, Langdon QK, Ohkuma M, Endoh R, Takashima M, Manabe R, Čadež N, Libkind D, Rosa CA, DeVirgilio J, Hulfachor AB, Groenewald M, Kurtzman CP, Hittinger CT, Rokas A (2018) Tempo and Mode of Genome Evolution in the budding yeast Subphylum. Cell 175:1533–1545e20. https://doi.org/10.1016/j.cell.2018.10.023

Simões ACP, Fernandes RP, Barreto MS, da Costa GBM, de Godoy MG, Freire DMG, Pereira N (2022) Growth of *Methylobacterium organophilum* in methanol for the simultaneous production of single-cell protein and metabolites of interest. Food Technol Biotechnol 60:338–349. https://doi.org/10.17113/ftb.60.03.22.7372

Singh HB, Kang MK, Kwon M, Kim SW (2022) Developing methylotrophic microbial platforms for a methanol-based bioindustry. Front Bioeng Biotechnol 10:1–18. https://doi.org/10.3389/fbioe.2022.1050740

Šmejkalová H, Erb TJ, Fuchs G (2010) Methanol assimilation in *Methylobacterium extorquens* AM1: demonstration of all enzymes and their regulation. PLoS ONE. https://doi.org/10.1371/journal.pone.0013001

Stratilová E, Breierová E, Vadkertiová R, Machová E, Malovíková A, Sláviková E (1998) The adaptability of the methylotrophic yeast *Candida boidinii* on media containing pectic substances. Can J Microbiol 44:116–120. https://doi.org/10.1139/cjm-44-2-116

Tomàs-Gamisans M, Ferrer P, Albiol J (2018) Fine-tuning the *P. pastoris* iMT1026 genome-scale metabolic model for improved prediction of growth on methanol or glycerol as sole carbon sources. Microb Biotechnol 11:224–237. https://doi.org/10.1111/1751-7915.12871

Van Uden N (1985) Temperature profiles of yeasts. Adv Microb Physiol 25:195–251. https://doi.org/10.1016/S0065-2911(08)60293-3

Wakayama K, Yamaguchi S, Takeuchi A, Mizumura T, Ozawa S, Tomizuka N, Hayakawa T, Nakagawa T (2016) Regulation of intracellular formaldehyde toxicity during methanol metabolism of the methylotrophic yeast *Pichia methanolica*. J Biosci Bioeng 122:545–549. https://doi.org/10.1016/j.jbiosc.2016.03.022

Wang Y, Fan L, Tuyishime P, Liu J, Zhang K, Gao N, Zhang Z, Ni X, Feng J, Yuan Q, Ma H, Zheng P, Sun J, Ma Y (2020a) Adaptive laboratory evolution enhances methanol tolerance and conversion in engineered *Corynebacterium glutamicum*. Commun Biol. https://doi.org/10.1038/s42003-020-0954-9

Wang Y, Fan L, Tuyishime P, Zheng P, Sun J (2020b) Synthetic methylotrophy: a practical solution for methanol-based biomanufacturing. Trends Biotechnol 38:650–666. https://doi.org/10.1016/j.tibtech.2019.12.013

Wang J, Liao Y, Qin J, Ma C, Jin Y, Wang X, Chen K, Ouyang P (2023a) Increasing lysine level improved methanol assimilation toward butyric acid production in *Butyribacterium methylotrophicum*. Biotechnol Biofuels Bioprod 16:10. https://doi.org/10.1186/s13068-023-02263-w

Wang J, Qin R, Guo Y, Ma C, Wang X, Chen K, Ouyang P (2023b) Engineering the native methylotrophs for the bioconversion of methanol to value-added chemicals: current status and future perspectives. Green Chem Eng 4:199–211. https://doi.org/10.1016/j.gce.2022.10.005

Wefelmeier K, Schmitz S, Haut AM, Otten J, Jülich T, Blank LM (2023) Engineering the methylotrophic yeast *Ogataea polymorpha* for lactate production from methanol. Front Bioeng Biotechnol 11:1–16. https://doi.org/10.3389/fbioe.2023.1223726

Wefelmeier K, Schmitz S, Kösters BJ, Liebal UW, Blank LM (2024) Methanol bioconversion into C3, C4, and C5 platform chemicals by the yeast *Ogataea Polymorpha*. Microb Cell Fact 23:1–15. https://doi.org/10.1186/s12934-023-02283-z

Wegat V, Fabarius JT, Sieber V (2022) Synthetic methylotrophic yeasts for the sustainable fuel and chemical production. Biotechnol Biofuels Bioprod 15:113. https://doi.org/10.1186/s13068-022-02210-1

Yurimoto H, Sakai Y (2019) Methylotrophic yeasts: current understanding of their C1-metabolism and its regulation by sensing methanol for survival on plant leaves. Curr Issues Mol Biol 33:197–209. https://doi.org/10.21775/CIMB.033.197

Yurimoto H, Oku M, Sakai Y (2011) Yeast methylotrophy: metabolism, gene regulation and peroxisome homeostasis. Int J Microbiol. https://doi.org/10.1155/2011/101298

Zaky AS, Greetham D, Louis EJ, Tucker GA, Du C (2016) A new isolation and evaluation method for marine- derived yeast spp. with potential applications in industrial biotechnology. J Microbiol Biotechnol 26:1891–1907. https://doi.org/10.4014/jmb.1605.05074

Zhan C, Li X, Yang Y, Nielsen J, Bai Z, Chen Y (2021) Strategies and challenges with the microbial conversion of methanol to high-value chemicals. Biotechnol Bioeng 118:3655–3668. https://doi.org/10.1002/bit.27862

Zhang W, Song M, Yang Q, Dai Z, Zhang S, Xin F, Dong W, Ma J, Jiang M (2018) Current advance in bioconversion of methanol to chemicals. Biotechnol Biofuels 11:1–11. https://doi.org/10.1186/s13068-018-1265-y

Zhang C, Ottenheim C, Weingarten M, Ji LH (2022a) Microbial utilization of Next-Generation feedstocks for the biomanufacturing of value-added chemicals and food ingredients. Front Bioeng Biotechnol 10:1–22. https://doi.org/10.3389/fbioe.2022.874612

Zhang S, Zhang W, Jiang M (2022b) Efficient fatty acid synthesis from methanol in methylotrophic yeast. Synth Syst Biotechnol 7:1183–1184. https://doi.org/10.1016/j.synbio.2022.09.003

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